

Odyssey

Infrared Imaging System



**Western
Blotting Methods**

Odyssey™ Western Blotting Protocols

Odyssey™ reagents: IR-labeled secondary antibodies
Odyssey™ blocking buffer

Additional reagents needed: Blotted nitrocellulose or
PVDF membrane
Primary antibody
Tween-20
PBS wash buffer
Double distilled water
Methanol for wetting of PVDF

Nitrocellulose or PVDF membranes may be used for protein blotting, but nitrocellulose membrane is recommended when maximum performance is desired. Pure cast nitrocellulose is generally preferable to supported nitrocellulose. Protein should be transferred from gel to membrane by standard procedures. After transfer:

1. Wet the membrane in PBS for several minutes. If using a PVDF membrane that has been allowed to dry, pre-wet briefly in 100% methanol and rinse with double distilled water before incubating in PBS.
2. Block the membrane in Odyssey blocking buffer for 1 hour. Be sure to use sufficient blocker (0.4 ml/cm² is suggested).
 - Membranes can be blocked overnight at 4°C if desired.
 - DO NOT add Tween-20 when blocking the membrane. The membrane should not be exposed to Tween-20 until blocking is completed, or high background will result.
 - Odyssey blocking reagent will yield higher and more consistent sensitivity and performance than other blockers. Nonfat dry milk or casein dissolved in PBS can also be used for blocking, but be aware that milk will cause higher background on PVDF membranes.

- Milk-based reagents can interfere with detection when using anti-goat antibodies. They also deteriorate rapidly at 4°C, so diluted antibodies cannot be kept and re-used for more than a few days.
- Odyssey blocker can often be diluted at least 1:1 in PBS without loss of performance.
- **Never block with BSA or BSA-containing solutions;** they cause very high background on the Odyssey imager.

3. Dilute the primary antibody in Odyssey blocker. The optimum dilution will depend on your antibody, and should be determined empirically. A suggested starting range is 1:1000 – 1:5000.
 - For nitrocellulose membranes, add 0.1-0.5% Tween-20 to the diluted antibody before incubation to lower background. The optimum Tween-20 concentration will depend on your antibody.
 - For PVDF membranes, excess Tween-20 may cause increased membrane background, particularly if blocking with milk. It may be best to avoid Tween-20 when using a milk-based blocker. If using Odyssey blocker, 0.1-0.2% Tween-20 can be added.
4. Incubate blot in primary antibody for 60 minutes or longer with gentle shaking (optimum incubation times will vary for different primary antibodies). Use enough antibody solution to completely cover the membrane.
5. Wash membrane 4 x 5 minutes in PBS + 0.1% Tween-20 with gentle shaking, using a generous amount of buffer.

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6. Dilute the fluorescently-labeled secondary antibody in Odyssey blocker. Avoid prolonged exposure of antibody vial to light. Suggested dilution range is 1:2000 – 1:10,000. Add Tween-20 to the diluted antibody as you did for the primary antibody.
 - For detection of very small amounts of protein (<50 pg), try using more secondary antibody (1:1000-1:2000).
 - Be careful not to introduce contamination into the antibody vial.
 - Diluted secondary antibody can be saved and re-used. Store at 4°C and protect from light. For highest sensitivity, use freshly diluted antibody solution.
7. Incubate blot in secondary antibody for 60 minutes with gentle shaking. Protect from light during the incubation.
 - Allowing incubation to proceed more than 60 minutes may increase background.
8. Wash membrane 4 x 5 minutes in PBS + 0.1% Tween-20 with gentle shaking. Protect from light.
9. Rinse membrane with PBS to remove residual Tween-20. The membrane is now ready to scan.
 - Scan in the appropriate channels (700 nm for Cy5.5 antibody, 800 nm for IRDye800™ antibody).
 - Protect the membrane from light until it has been scanned.
 - Keep the membrane wet if you plan to strip and re-use it. Once a membrane has dried, stripping will be ineffective.
 - Blots can be allowed to dry before scanning if desired. Signal strength may be enhanced on a dry membrane. The membrane can also be re-wet for scanning.

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- The fluorescent signal on the membrane will remain stable for several weeks or longer if protected from light. Membranes may be stored dry or in PBS buffer at 4°C.
- If signal on membrane is too strong, re-scan the membrane at a lower intensity setting.

Molecular Weight Marker

If you loaded the Odyssey Prestained Molecular Weight Marker on your gel before transfer, it will be visible on the blot in the 700 nm (Cy5.5) channel, and also faintly visible in the 800 nm channel. If the marker is subjected to numerous freeze/thaw cycles, it may degrade. This is observed as multiple, high-molecular weight bands appearing in the 800 nm channel. If this occurs, discard the aliquot and use a fresh one.

Two-Color Western Blots

Two different antigens can be detected simultaneously on the same blot using IRDye800™ and Cy5.5 antibodies. For information about choosing appropriate antibodies for two-color blotting, see *Guidelines For Two-Color Western Blot Applications*.

When performing a two-color blot, use the standard Western blot protocol, with the following modifications:

- Combine the two primary antibodies in the diluted antibody solution in step 3. Incubate simultaneously with membrane.
- Combine the two dye-labeled secondary antibodies in the diluted antibody solution in step 6. Incubate simultaneously with membrane.
- Make sure antibody specificities are appropriate, and that each labeled secondary chosen is labeled with a different dye.

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Alternate Protocol: Tertiary Detection

In some cases, you may wish to amplify the signal on a Western blot to achieve greater sensitivity. This can be accomplished by adding another antibody to the detection process, a tertiary antibody. Tertiary detection will increase the detection sensitivity two- to four-fold over the standard protocol. It is usually performed with an unlabeled secondary antibody (goat anti-mouse, for example) and a dye-labeled tertiary antibody (IRDye800 donkey anti-goat).

The protocol can also be performed using secondary and tertiary antibodies that are both labeled with dye, but this often causes higher background. Note that two-color blotting cannot be performed when using tertiary detection (the IRDye800 donkey anti-goat tertiary antibody will react against any goat-derived secondary antibodies bound to the blot).

For tertiary detection, make the following modifications to the standard protocol:

- Perform steps 1-5 as you normally would.
- After washing the blot in step 5, skip to the alternate protocol below (steps 6a - 12a):

Alternate protocol:

- 6a.** Dilute unlabeled secondary antibody in Odyssey blocker. Because sensitivity is important, the suggested dilution range for unlabeled secondary is 1:1000 - 1:2500. Add Tween-20 to the diluted antibody as you did for the primary antibody.
- 7a.** Incubate blot in secondary antibody for 60 minutes or longer with gentle shaking.

- 8a.** Wash membrane 4 x 5 minutes in PBS + 0.1% Tween-20 with gentle shaking.
- 9a.** Dilute IRDye800-labeled tertiary antibody in Odyssey blocker. The suggested dilution range for labeled tertiary is 1:1000 - 1:2500. Optimize tertiary dilution for best results. Add Tween-20 to the diluted antibody as you did for the primary antibody.
- 10a.** Incubate blot in tertiary antibody for 60 minutes with gentle shaking. Protect from light during the incubation.
- 11a.** Wash membrane 4 x 5 minutes in PBS + 0.1% Tween-20 with gentle shaking. Protect from light.
- 12a.** Rinse membrane with PBS to remove residual Tween-20. The membrane is now ready to scan.

Stripping of the membrane

- PVDF membranes may be stripped. Nitrocellulose generally does not strip well.
- Wash membrane for 30 minutes in 25 mM glycine-HCl pH 2.0 + 1% SDS with gentle shaking at room temperature.
- Rinse in PBS for 10 minutes.
- Quickly scan membrane at low resolution to determine if stripping was successful. Repeat glycine/SDS wash and PBS rinse if necessary.

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Optimization Tips

- Adhere carefully to recommendations regarding blocking methods and reagents.
- To avoid background speckles on blots, use high-quality double distilled water for buffers and rinse plastic dishes well before and after use. Membranes should be handled only by their edges, with forceps.
- After you handle membranes that have been incubating in diluted secondary antibody, clean forceps thoroughly with distilled water and/or ethanol. If forceps are not cleaned after being dipped in antibody solution, they can cause spots or streaks of background on the membrane that will not wash away.
- When scanning, always clean the instrument glass to remove dust and smudges that may affect the quality of your image. If using a silicon mat over your membranes, carefully clean the surface that will touch the membrane. A dirty mat can deposit dust and smudges that cannot be washed away.
- If you plan to strip a Western blot, do not allow the membrane to dry. Once a membrane has dried, stripping will be ineffective.

Guidelines For Two-Color Western Blot Applications

For simultaneous detection of two antigens, the primary and secondary antibodies must be carefully selected to meet the following criteria:

- **The two primary antibodies must be derived from different host species** so they can be discriminated by secondary antibodies of different specificities (for example, primaries from rabbit and mouse will be discriminated by anti-rabbit and anti-mouse secondary antibodies).

- Always perform preliminary blots with each primary antibody alone to determine the expected banding pattern for each, before combining them in a two-color experiment. Slight cross-reactivity may occur, particularly if the antigen is very abundant, and can complicate interpretation of your blot. If cross-reactivity is a problem, load less protein or reduce the amount of antibody.
- For best results, avoid using primary antibodies from mouse and rat together for a two-color experiment. Because the species are so closely related, it is not possible to completely adsorb away cross-reactivity. Substantial cross-reactivity between bands may occur. If using mouse and rat together, it is crucial to run single-color blots first with each individual antibody to be certain of expected band sizes.
- **The two secondary antibodies used must be derived from the same host species** (goat) so that they will not react against one another. The secondary antibodies should not recognize immunoglobulins from other species that may be present in the sample.
- One secondary antibody must be labeled with IRDye800, and the other with Cy5.5.
- When using tertiary detection with donkey anti-goat to amplify signal, two-color detection cannot be performed. Donkey anti-goat will recognize all goat-derived secondary antibodies that are present on the blot.

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Optimization of Antibody Concentration

When adapting your Western blotting protocols for Odyssey detection or using a new primary antibody, it is important that you determine the optimal antibody concentrations. Optimization will allow you to achieve maximum sensitivity and consistency.

Three parameters should be optimized:

1. Primary antibody concentration
2. Dye-labeled secondary antibody concentration
3. Tween-20 concentration in diluted antibody

Optimal primary antibody concentrations vary widely, depending on the affinity and avidity of the antibody in question, and also the detection sensitivity required in the experiment. The correct secondary antibody concentration depends on the performance of the primary antibody and the required sensitivity.

Tween-20 concentration also plays a role. Addition of Tween-20 to diluted primary and secondary antibodies can substantially reduce background, leading to improved detection sensitivity. However, too much Tween-20 can wash away bound antibody and potentially wash away antigen from the blot. To maximize performance of your Western system, you should test several dilutions of primary and secondary antibodies, as well as several Tween-20 concentrations.

Primary Antibody Optimization

Primary antibodies vary widely, in reactivity and in concentration. The correct working range for antibody dilution will depend on the characteristics of your primary antibody and the amount of target antigen you wish to detect. Suggested dilutions to test are 1:500, 1:1500, 1:5000, and 1:10,000 (start where you normally would for chemiluminescent detection). Optimize your primary dilution to achieve maximum performance and to conserve antibody.

Secondary Antibody Optimization

Optimal dilutions of Odyssey dye-conjugated secondary antibodies should also be determined. Suggested starting dilutions to test are 1:1500, 1:5000, and 1:10,000. The amount of secondary required will vary depending on how much antigen is being detected – abundant proteins with strong signals will require less secondary antibody. Optimal Odyssey detection may require more secondary antibody than you would use for chemiluminescence. After incubation with the blot, the diluted antibody can be saved and re-used several times (if stored at 4°C and protected from light). If Tween-20 precipitates, warm antibody solution to room temperature to redissolve the detergent. If high sensitivity is desired, however, dilute fresh antibody just before use.

Tween-20 Optimization

Addition of Tween-20 to diluted antibodies may significantly reduce background on the blot. The optimal detergent concentration should be determined for each primary antibody, because some primaries do not bind as tightly as others and may be washed away by too much Tween-20. The proper amount of Tween-20 also depends on the choice of blotting membrane. See following guidelines to determine the appropriate detergent concentration. **Never expose any membrane to Tween-20 until after blocking has been completed. Tween-20 will stick to the bare membrane and cause very high background.**

For nitrocellulose membrane: Suggested concentration ranges from 0.05 - 0.5% Tween-20; a good starting concentration is 0.1 - 0.2%. (Note that if your primary can tolerate increased detergent (up to 1%), this may reduce background and improve your sensitivity. However, increased detergent can cause antigen to be lost from the blot in some cases.)

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For PVDF membrane: On this type of membrane, Tween-20 will somewhat increase background, particularly if you are blocking with nonfat dry milk. It may be best to avoid any Tween-20 in diluted antibody if you are using a milk blocker. Odyssey Blocker will give much better performance for antibody dilution on PVDF, and 0.1 - 0.2% Tween-20 can be added.

A dot blot assay format is convenient for determining optimal antibody concentrations. Prepare dilutions of the sample in PBS or TBS buffer. To find the best working range for your antibody system, it is helpful to test a wide range of dilutions. Sample dilutions can range from microgram to picogram levels, depending on the concentration of antigen in your sample. Spot 1-2 µl of each protein dilution onto a dry nitrocellulose membrane. Allow the membrane to air-dry completely (at least 30 minutes) before proceeding with blocking and detection. Multiple narrow strips of nitrocellulose can easily be processed in parallel using individual conical or round-bottomed capped plastic tubes for antibody incubations.

Remember...

- **Do not** expose your membrane to Tween-20 until after it has been blocked. The presence of Tween-20 during blocking will cause strong, irreversible background fluorescence on the blot.
- **Never** use BSA or BSA-containing solutions for blocking or antibody dilution in the Odyssey system. Incubating the membrane with BSA causes very high background.
- Nonfat dry milk works well for nitrocellulose, but when using PVDF membrane, **stick with the Odyssey Blocker** for best results.

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- Milk-based blockers may contain IgG that will react with anti-goat antibodies. This can significantly increase background and reduce antibody titer. **Always use** Odyssey Blocker with anti-goat antibodies.
- To stretch the Odyssey blocker: **recycle** your blocking buffer for antibody dilution; dilute 1:1 in PBS; save excess used blocker at 4°C for several days for re-use (use a separate container – don't return to the main bottle).
- When using PVDF, **do not exceed** ~0.2% Tween-20 in your diluted antibodies. Additional Tween-20 can increase background. This can be particularly severe when using milk for blocking and dilution.
- Diluted secondary antibody in Odyssey blocker can be **saved and re-used** (store at 4°C and protect from light). The Tween-20 may form a white precipitate during prolonged storage, which should re-dissolve when the solution is warmed to room temperature.
- Store the antibody vial at 4°C in the dark. **Minimize exposure to light**, and take care not to introduce contamination into the vial. Dilute immediately prior to use. If particulates are seen in the antibody solution, centrifuge before use.
- **Protect membrane from light** during secondary antibody incubations and washes.

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More Tips...

- Use the narrowest well size possible for your loading volume to concentrate the target protein.
- Use nitrocellulose membrane for transfer.
- Small amounts of purified protein may not transfer well. Addition of nonspecific proteins of similar molecular weight can have a "carrier" effect and substantially increase transfer efficiency.
- Take a look at transfer conditions – addition of SDS to the transfer buffer can greatly reduce the binding of transferred proteins to the membrane (for both PVDF and nitrocellulose). For proteins <100 kDa, try blotting in standard Tris-glycine buffer with 20% methanol and no SDS.
- Soak the gel in transfer buffer for 10-20 minutes before setting up the transfer. This equilibrates the gel and removes SDS from the gel so that it will not be carried over into the transfer tank.
- To maximize retention of transferred proteins on the membrane, allow the membrane to air-dry completely after transfer (~1-2 hours).
- Don't over-block. Long blocking incubations, particularly with nonfat dry milk at 2% or higher, can cause loss of target protein from the membrane (*J. Immunol. Meth.* 122:129-135, 1989).
- To improve the sensitivity of your primary antibody, try extended incubation at room temperature or overnight incubation at 4°C to enhance signal. Avoid extended incubations in secondary antibody.
- The best transfer conditions, membrane, and blocking agent for your experiment will vary, depending on the antigen and antibody. If you have problems with high background, try a different blocking agent; if low signal is the problem, consider optimizing your transfer conditions.

Imaging of Coomassie-stained protein gels

The Odyssey can also be used to document Coomassie-stained gels. Coomassie Blue dye can be seen clearly in the 700 nm channel, and faintly in the 800 nm channel. The Odyssey is often more sensitive than what you can visually see on the gel. To document a gel:

- Thoroughly rinse the gel with de-staining solution or water to remove dye particulates. Dye particles on the gel surface cause background speckles on the image.
- Place the gel on the scanner glass, taking care not to trap bubbles underneath. If desired, cover the gel with plastic wrap to prevent it from drying out.
- Scan the gel in the 700 nm channel. The focus offset must be adjusted for gel imaging. The correct focus offset is 1/2 the thickness of the gel; for a 1 mm gel, set the focus offset to 0.5 mm.
- After removing the gel, clean the glass surface carefully to remove any residual dye.
- If dye particulates still appear on the gel image, wipe the gel gently with a gloved finger or wet Kimwipe before scanning, or use the filter/noise removal software option to eliminate speckles.

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Troubleshooting Guide

Problem: High background, uniformly distributed

Possible Causes

Membrane blocked in presence of Tween-20

BSA used for blocking

Not using optimal blocking reagent

Background on nitrocellulose

Background on PVDF

Antibody concentrations too high

Insufficient washing

Cross-reactivity of antibody with contaminants in blocking buffer

Inadequate antibody volume used

Membrane contamination

Recommendation

Do not expose any membrane to Tween-20 until **after** blocking.

Never use BSA solutions for blocking or antibody dilution. BSA causes strong, irreversible background.

Compare different blocking buffers to find the most effective for your system; try blocking longer.
Use Odyssey blocker rather than milk or casein for more consistent performance.

Add Tween-20 to the diluted antibodies to reduce background. Tween-20 concentration should be optimized for your primary antibody, from 0.05 - 1%.

Reduce or eliminate Tween-20 in diluted antibody, particularly if using milk as blocker.

Optimize primary and secondary antibody dilutions.

Increase number of washes and buffer volume.
Make sure that 0.1% Tween-20 is present in buffer, and increase if needed. Note that excess Tween-20 (0.5-1%) may decrease signal.

Use Odyssey blocker, not milk. Milk is usually contaminated with IgG and will cross-react with anti-goat secondary antibodies.

Increase antibody volume so entire membrane surface is sufficiently covered with liquid at all times (use heat-seal bags if antibody volume is limiting). Do not allow any area of membrane to dry out.

Use agitation for all antibody incubations.

Always handle membranes carefully and with forceps. Do not allow membrane to dry.

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Problem: Uneven blotchy or speckled background

Possible Causes

Blocking multiple membranes together in small volume

Membrane stained with Ponceau S

Membrane not fully wetted or allowed to partially dry

Contaminated forceps or dishes

Recommendation

If multiple membranes are being blocked in the same dish, ensure that blocker volume is adequate for all membranes to move freely and make contact with liquid.

Pre-staining of membranes with Ponceau S may cause increased background even after stain is washed away.

Keep membrane completely wet at all times. This is particularly crucial if blot will be stripped and re-used.

If using PVDF, remember to pre-wet in 100% methanol first.

Always carefully clean forceps after they are dipped into an antibody solution, particularly dye-labeled secondary antibody. Dirty forceps can deposit dye on the membrane that will not wash away.

Use clean dishes, bags or trays for incubations.

Problem: Weak or no signal

Proteins did not transfer well out of gel

Protein lost from membrane during detection

Insufficient antigen used

Wrong blocker used

Check transfer buffer choice and blotting procedure for your gel/blotter system.

Use Odyssey pre-stained molecular weight marker to monitor transfer, and stain gel after transfer to make sure proteins are not retained in gel.

Extended blocking times or high concentrations of Tween-20 (0.5-1%) in antibody dilutions may lead to some loss of antigen from a blotted membrane.

Load more protein on the gel; try using the narrowest possible well size to concentrate antigen.

For increased sensitivity and consistency, use Odyssey blocker.

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Troubleshooting Guide (continued)

Problem: Weak or no signal (continued)

Possible Causes

Proteins not retained on membrane during transfer

Recommendation

Allow membrane to air-dry completely after transfer (1-2 hours) before blocking step. This helps make protein binding irreversible.

Optimize transfer conditions and time for your antigen. Check for bubbles in transfer sandwich.

Try a different brand or type of membrane (binding to membrane may vary for different antigens).

Addition of 20% methanol to transfer buffer may improve binding of antigen on membrane (*note: methanol decreases pore size of gel and can hamper transfer of large proteins*).

Presence of SDS in the transfer buffer may reduce the binding of transferred proteins to nitrocellulose or PVDF, especially for low molecular weight proteins. Try reducing or eliminating SDS (*note: presence of up to 0.05% SDS does improve transfer efficiency of some proteins from gel*).

Small proteins may pass through membrane during transfer ("blow-through"). Use a membrane with a smaller pore size or reduce transfer time.

Insufficient antibody used

Primary antibody may be of low affinity. Increase amount of antibody, optimizing concentration for best performance.

Extend primary antibody incubation (4-8 hrs. at room temperature, or overnight at 4°C).

Increase amount of secondary antibody, optimizing for best performance.

Antibody may have lost reactivity due to age or improper storage; test antibody with a dot blot.

Wrong membrane type

Try a different membrane. Use pure nitrocellulose membrane, not PVDF, for greatest sensitivity.

Problem: Nonspecific or unexpected bands

Possible Causes

Antibody concentrations too high

Recommendation

Reduce the amount of antibody used.

Reduce antibody incubation times.

Increase Tween-20 in diluted antibodies.

Cross-reactivity between antibodies in a two-color experiment

Double-check the sources and specificities of the primary and secondary antibodies used (see Guidelines for Two-Color Westerns).

There is always potential for cross-reactivity in a two-color experiment. Use less secondary antibody to minimize this.

Always test the two colors on separate blots first so you know what bands to expect and where.

Avoid using mouse and rat antibodies together, if possible. Because the species are so closely related, anti-mouse will react with rat IgG to some extent, and anti-rat with mouse IgG.

Bleedthrough of signal from one channel into other channel

If signal in one channel is very strong (near or at saturation) it may generate a small amount of bleedthrough signal in the other channel. Minimize this by using a lower intensity setting for scanning in the problem channel.

Reduce signal in further experiments by reducing the amount of protein loaded or antibody used.

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